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Note

High-performance thin-layer chromatography of diastereomeric di- and tripeptides on ready-for-use plates of silanized silica gel and on ammonium tungstophosphate layers

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Home-made and ready-for-use plates of silanized silica gel, untreated or impregnated with anionic and cationic detergents, and layers of synthetic inorganic exchangers, such as ammonium tungstophosphate, showed a high efficiency in the separation of di-, tri- and polypeptides[1–5]. It was of interest, therefore, to determine the optimum conditions for the separation of diastereoisomeric di- and tripeptides on these plates and to establish some general criteria for the resolution of more complex mixtures.

The separation of some diastereomeric dipeptides has been effected on thin layers of silica gel[6,7] and of microcrystalline cellulose[6], and a larger number of optical isomers have been separated on columns of silanized silica gel[8,9].

EXPERIMENTAL

The standard solutions of the peptides (1 mg/ml) were prepared by dissolving the compounds in water–methanol (1:1).

The sample volume was 0.2 μl in the case of silanized silica gel untreated or impregnated with anionic and cationic detergents, and 0.5 μl for ammonium tungstophosphate. The spots were visualized by spraying with a 1% ninhydrin solution in pyridine–acetic acid (5:1) and heating the plates for 5 min at 100°C.

The Sil C_{18}-50 (Macherey, Nagel & Co., Düren, G.F.R.) and the OPTI-UP C_{12} (Antec, Bennwil, Switzerland) plates were impregnated as previously described[3], and the ammonium tungstophosphate (AWP) layers were prepared as before[2,5]. The migration distance was 6 cm for the ready-for-use plates and 10 cm for the AWP layers, unless otherwise stated. The chromatographic measurements were carried out at 25°C.

RESULTS AND DISCUSSION

Untreated layers of silanized silica gel

The behaviour of the diastereomers was examined on commercially available layers of silanized silica gel, OPTI-UP C_{12}, Sil C_{18}-50 and RP-18. The RP-18 plates are ill-suited, as eluents with at least 60% methanol must be used and under these elution conditions most peptides are very weakly retained. Table I lists the chro-

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TABLE I

R_F VALUES OF PEPTIDE DIASTEREOMERS ON PLATES OF OPTI-UP C_{12} AND SIL C_{18}-50 UNTREATED OR IMPREGNATED WITH 4% HDBS IN DIFFERENT ELUENTS

Eluents: 1, 1 *M* acetic acid in water; 2, 1 *M* acetic acid + 3% KCl in water; 3, 0.5 *M* sodium acetate in water; 4, 1 *M* acetic acid in water–methanol (30%); 5, 1 *M* acetic acid + 0.1 *M* HCl in water–methanol (20%); 6, 1 *M* acetic acid + 1 *M* HCl in water–methanol (20%).

Peptide	*OPTI-UP C_{12}*			*SIL C_{18}-50,*	*SIL C_{18}-50 + 4% HDBS*	
	1	*2*	*3*	*4*	*5*	*6*
L-Ala-L-Ala	0.94	0.95	0.91	0.96	0.75	0.80
D-Ala-L-Ala	0.83	0.83	0.80	0.96	0.68	0.80
L-Ala-D-Ala	0.83	0.83	0.80	0.96	0.68	0.80
D-Ala-D-Ala	0.94	0.95	0.91	0.96	0.74	0.80
L-Ala-L-Ala-L-Ala	0.87	0.87	0.82	0.96	0.75	0.81
L-Ala-L-Ala-D-Ala	0.74	0.77	0.72	0.96	0.68	0.80
L-Ala-D-Ala-L-Ala	0.63	0.69	0.57	0.96	0.64	0.79
D-Ala-D-Ala-D-Ala	0.87	0.87	0.82	0.96	0.75	0.81
Gly-L-Ala	0.94	0.95	0.92	0.96	0.76	0.84
Gly-D-Ala	0.94	0.95	0.92	0.96	0.76	0.84
L-Leu-Gly	0.54	0.57	0.41	0.82	0.25	0.55
D-Leu-Gly	0.54	0.57	0.41	0.82	0.25	0.55
L-Ala-L-Leu	0.45	0.52	0.53	0.80	0.16	0.35
D-Ala-L-Leu	0.33	0.35	0.30	0.71	0.12	0.29
L-Leu-L-Leu	0.16	0.18	0.10	0.56	0.05	0.17
D-Leu-L-Leu	0.06	0.06	0.04	0.34	0.00	0.06
L-Leu-D-Leu	0.06	0.06	0.04	0.34	0.00	0.06
D-Leu-D-Leu	0.16	0.18	0.10	0.56	0.05	0.17
L-Leu-L-Tyr	0.26	0.29	0.18	0.70	0.13	0.33
D-Leu-L-Tyr	0.23	0.23	0.12	0.66	0.08	0.30
L-Tyr-L-Arg	0.47	0.58	0.19	0.82	0.01	0.36
L-Tyr-D-Arg	0.45	0.54	0.13	0.79	0.01	0.33

matographic characteristics of the peptides on OPTI-UP C_{12} and Sil C_{18}-50 plates eluted with aqueous solutions at different pH values and ionic strengths (see columns 1–3) or with a 30% methanol content (column 4). As in column chromatography[9], in an acidic medium a sharp separation of L–L and D–D enantiomers from L–D and D–L is observed. These last compounds are more strongly retained in accordance with their greater hydrophobic characteristics produced by the *cis* disposition of the alkyl groups of the amino acid residues. This behaviour is also common to the tripeptides, Ala_3, where the R_F sequence L–L–L > L–L–D > L–D–L is opposite to that of their hydrophobicity. The presence in the dipeptides of a Gly sub-unit results in equal R_F values for the two optical isomers.

An increase of the ionic strength of the eluent, obtained by addition of 3% KCl to the 1 *M* acetic acid solution (column 2), sometimes improves the resolution of the diastereomers, as evidenced by the R_F values of the dipeptides containing arginine.

In alkaline solutions a slightly more marked retention of most peptides and, in some cases, a better resolution of the diastereomers are observed. In particular, with 0.5 *M* sodium acetate aqueous solution, pH 8.15 (column 3), as eluent the

diastereomers of the Ala_3 tripeptide are well-separated and the resolution of those containing tyrosine is further improved.

These results cannot be compared with those obtained on column chromatography since in this case eluents at pH < 8 must be used[9]. On the one hand the presence of methanol in the eluent (see column 4, Sil C_{18}-50 plates) results in a poorer resolution of the diastereomers formed by one or more polar sub-units, on the other it improves the separation of those formed by non-polar sub-units, *i.e.*, Leu-Leu. These trends are observed also in an alkaline medium. On Sil C_{18}-50 plates, eluted with 0.5 *M* sodium acetate in 20% methanol, the two diastereomers of Leu-Tyr and Tyr-Arg exhibit the same R_F values (0.36 for the first pair and 0.55 for the second) notwithstanding their higher retention with respect to the acidic medium.

From an analytical point of view the best results are obtained on OPTI-UP C_{12} plates, where aqueous eluents can be used (see Fig. 1). The elution time is extraordinarily low (6–7 min for a migration distance of 6 cm). However, not very compact spots, are obtained, preventing the separation of the two diastereomers L-Leu-L-Tyr and D-Leu-L-Tyr whose R_F values only differ by 0.03 units. Nevertheless this separation may be effected in an alkaline medium.

Sil C_{18}-50 impregnated with 4% dodecylbenzenesulphonic acid (HDBS) or 4% N-dodecylpyridinium chloride (N-DPC)

Only the Sil C_{18}-50 plates were impregnated since on OPTI-UP C_{12} the detergents are so weakly adsorbed that during elution with aqueous or aqueous–organic solutions they migrate giving rise to irregular solvent fronts.

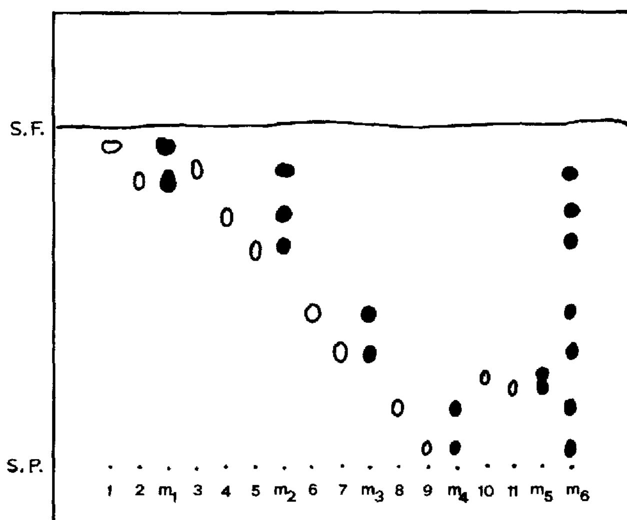


Fig. 1. Thin-layer chromatogram of diastereomers on UP C_{12} plates. Eluent: 1 *M* acetic acid in water. Peptides: 1 = L-Ala-LAla; 2 = L-Ala-D-Ala; 3 = L-Ala-L-Ala-L-Ala; 4 = L-Ala-L-Ala-D-Ala; 5 = L-Ala-D-Ala-L-Ala; 6 = L-Ala-L-Leu; 7 = D-Ala-L-Leu; 8 = L-Leu-L-Leu; 9 = D-Leu-L-Leu; 10 = L-Leu-L-Tyr; 11 = D-Leu-L-Tyr; m_1 = mixture of 1 and 2; m_2 = mixture of 3–5; m_3 = mixture of 6 and 7; m_4 = mixture of 8 and 9; m_5 = mixture of 10 and 11; m_6 = mixture of 3–9. S.P. = starting point; S.F. = solvent front.

The presence of the anionic detergent (HDBS) on the layer results in a higher retention of the peptides (see columns 5 and 6 of Table I) and in the formation of very compact and spherical spots. This allows the separation of diastereomers, even when the difference in their R_F values is only 0.03 units (see Fig. 2). Concerning the retention mechanism, few compounds give rise to linear R_M/apparent pH trends, with slopes lower than the theoretical ones, supporting the presence of a cation-exchange process which is not, however, prevailing with respect to the partition process.

On layers of Sil C_{18}-50 impregnated with the cationic detergent (N-DPC) interesting results are obtained in an alkaline medium. When eluting with 0.5 *M* sodium acetate in water–methanol (20%), the diastereomeric dipeptides formed by a tyrosine sub-unit can readily be separated (see Fig. 3); in contrast, such compounds may be separated only with difficulty on layers of OPTI-UP C_{12} and of Sil C_{18}-50 impregnated with HDBS.

The reversal of the retention order of these diastereomers with respect to that observed on the same layer in the absence of detergent seems related to the presence of a predominantly anion-exchange process. This is not the case under acidic conditions since the dipeptides are in their cationic forms. As the structures of the two diastereomers of Leu-Tyr show, the ion-exchange process is favoured by the greater distance between the two polar groups ($-NH_2$ and $-COO^-$) in the L–L isomer compared with the L–D one:

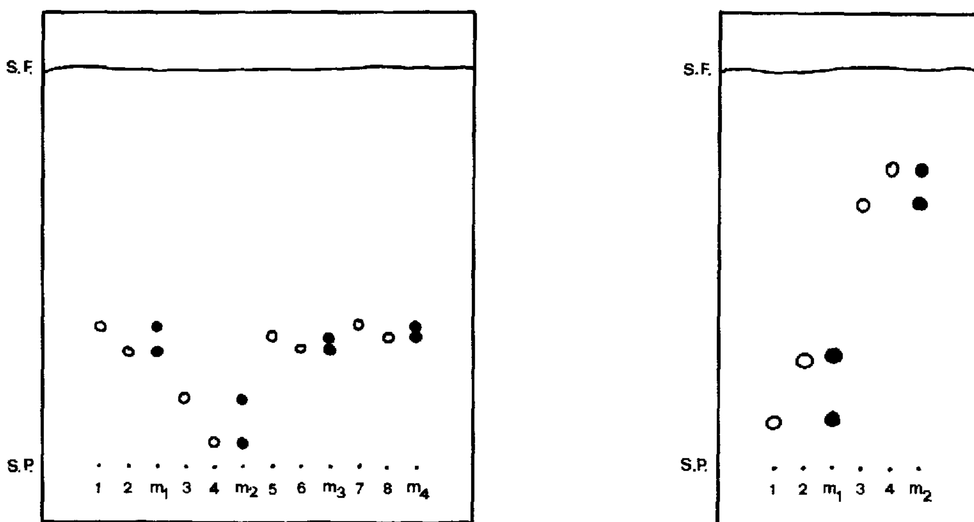
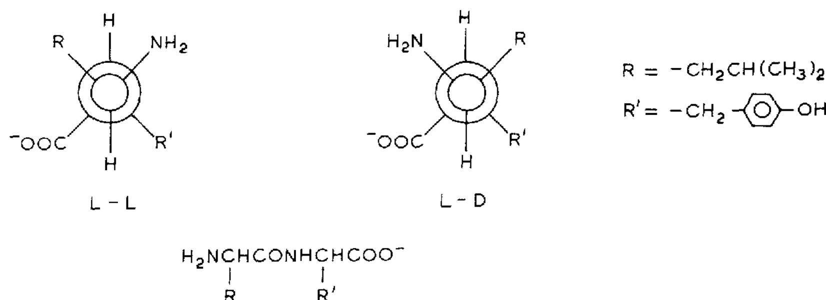


Fig. 2. Thin-layer chromatogram of diastereomers on Sil C_{18}-50 plates impregnated with 4% HDBS. Eluent: 1 *M* acetic acid + 1 *M* hydrochloric acid in water–methanol (20%). Migration distance = 7 cm. Peptides: 1 = L-Ala-L-Leu; 2 = D-Ala-L-Leu; 3 = L-Leu-L-Leu; 4 = D-Leu-L-Leu; 5 = L-Leu-L-Tyr; 6 = D-Leu-L-Tyr; 7 = L-Tyr-L-Arg; 8 = L-Tyr-D-Arg. m_1 = mixture of 1 and 2; m_2 = mixture of 3 and 4; m_3 = mixture of 5 and 6; m_4 = mixture of 7 and 8.

Fig. 3. Thin-layer chromatogram of diastereomers on Sil C_{18}-50 plates impregnated with 4% N-DPC. Eluent: 0.5 *M* sodium acetate in water–methanol (20%). Peptides: 1 = L-Leu-L-Tyr; 2 = D-Leu-L-Tyr; 3 = L-Tyr-L-Arg; 4 = L-Tyr-D-Arg; m_1 = mixture of 1 and 2; m_2 = mixture of 3 and 4.



The same phenomenon also accounts for the behaviour of the Tyr-Arg diastereomers.

Layers of ammonium tungstophosphate

Table II lists the chromatographic characteristics of the peptides eluted with ammonium nitrate solutions at different concentrations and with 0.5 *M* nitric acid. In all cases the tripeptides Ala_3 are more strongly retained than the dipeptides Ala_2 in accordance with the greater distance in the former between the $-NH_3^+$ group and the carboxylic group. This behaviour was not observed on plates of silanized silica gel untreated or impregnated with detergents, where the optical isomers of Ala_2 cannot be separated from those of Ala_3.

TABLE II

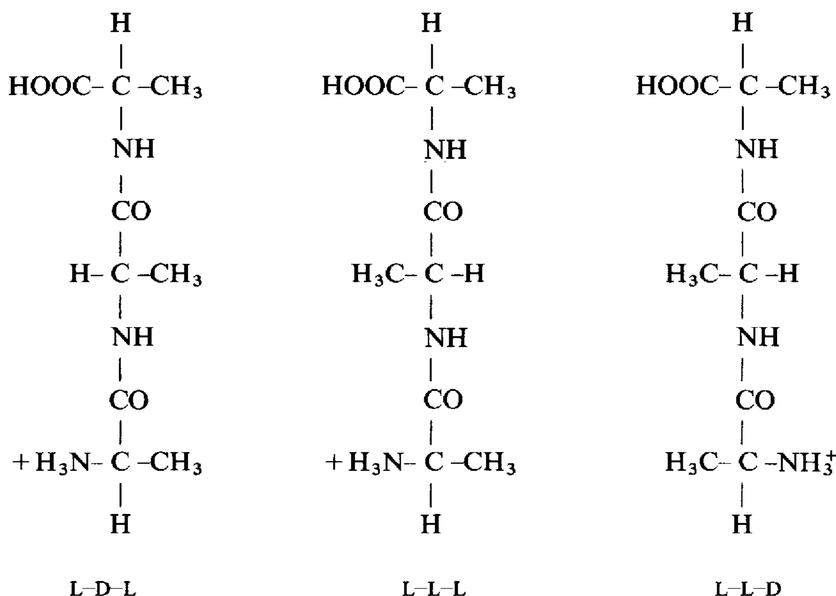
R_F VALUES OF PEPTIDE DIASTEREOMERS ON PLATES OF AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2) WITH DIFFERENT ELUENTS

Peptide	*Eluent*		
	1 M NH_4NO_3	*3 M NH_4NO_3*	*0.5 M HNO_3*
L-Ala-L-Ala	0.52	0.78	0.25
D-Ala-L-Ala	0.54	0.78	0.29
L-Ala-D-Ala	0.54	0.78	0.29
D-Ala-D-Ala	0.52	0.78	0.25
L-Ala-L-Ala-L-Ala	0.34	0.63	0.09
L-Ala-L-Ala-D-Ala	0.32	0.63	0.07
L-Ala-D-Ala-L-Ala	0.34	0.62	0.13
D-Ala-D-Ala-D-Ala	0.34	0.63	0.09
Gly-L-Ala	0.46	0.70	0.17
Gly-D-Ala	0.46	0.68	0.17
L-Leu-Gly	0.47	0.70	0.13
D-Leu-Gly	0.47	0.70	0.13
L-Ala-L-Leu	0.50	0.72	0.28
D-Ala-L-Leu	0.55	0.73	0.36
L-Leu-L-Leu	0.53	0.67	0.27
D-Leu-L-Leu	0.53	0.67	0.28
L-Leu-D-Leu	0.53	0.67	0.28
D-Leu-D-Leu	0.53	0.67	0.28
L-Leu-L-Tyr	0.31	0.54	0.10
D-Leu-L-Tyr	0.37	0.54	0.17
L-Tyr-L-Arg	0.04	0.18	0.00
L-Tyr-D-Arg	0.06	0.21	0.00
1st front	0.75	0.90	–

Another difference is the sequence reversal among the diastereomers; on AWP the L–L or D–D enantiomers are more strongly retained than the L–D and D–L ones. The *trans* position of the amino and carboxylic groups in the first isomers permits an easier exchange process between the $-NH_3^+$ group and the inorganic exchanger. In contrast, in the case of the Leu-Leu diastereomers no differences in the chromatographic behaviour are observed. This can be ascribed to the levelling effect of the hydrophobic side-chains of the amino acid residues on the interactions between the two polar groups, since on ammonium tungstophosphate layers the retention is not correlated to the hydrophobic characteristics of the molecules.

Plots of the R_M values as a function of the ammonium nitrate activity in the 0.5–3 *M* concentration range were linear for most compounds, with slopes between 0.5 and 1.1. This shows that the occurrence of an ion-exchange process must be considered. Such a process, however, is predominant only in a few cases since the values of the slopes are generally smaller than those predicted theoretically[10].

The behaviour of Ala_3 stereoisomers, eluted with 0.5 *M* nitric acid, is of particular interest. The retention order of the diastereomers, L-L-D > L-L-L > L-D-L, is completely different from that observed on silanized silica gel plates and can be explained on the basis of the following structures[9]:



The L–L–D stereoisomer is the most strongly retained owing to the *trans* position of the $-NH_3^+$ group and of the carboxyl group, while the L–D–L one is the least strongly retained since the polar groups are in the *cis* position separated by only an hydrogen atom, whose shielding effect is surely less than that of the methyl group in the L–L–L isomer. From the data of Table II it is seen that the resolution of the diastereomers improves in acidic solution, where the peptides are in the cationic form, with respect to the ammonium nitrate medium where the zwitterionic form prevails.

Fig. 4 shows some interesting separations concerning the Ala_2, Ala_3, Ala-Leu

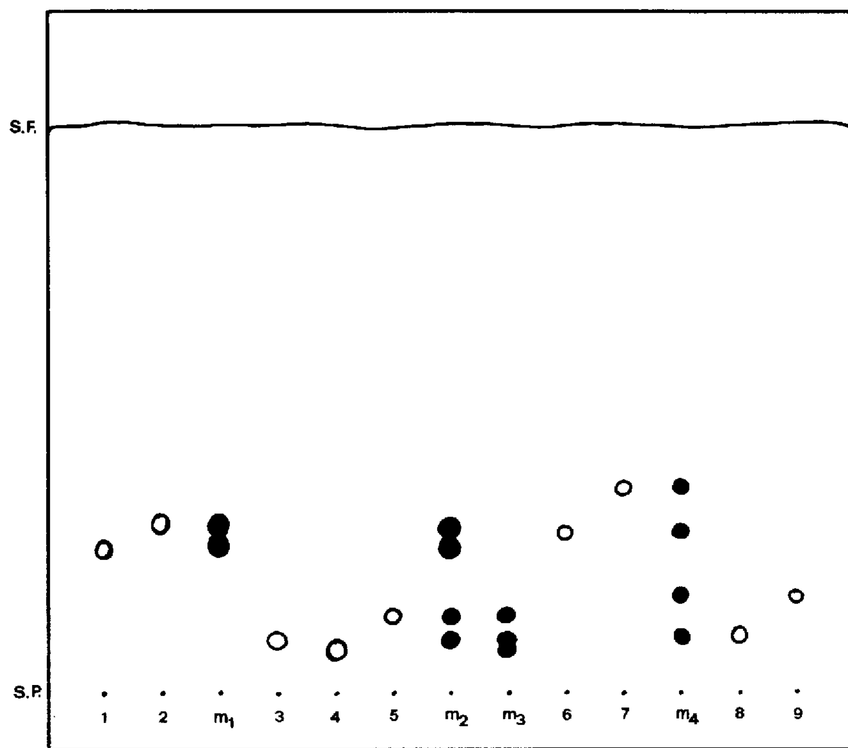


Fig. 4. Thin-layer chromatography of diastereomers on AWP + $CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2) plates. Eluent: 0.5 *M* nitric acid in water. Peptides: 1 = L-Ala-L-Ala; 2 = D-Ala-L-Ala; 3 = L-Ala-L-Ala-L-Ala; 4 = L-Ala-L-Ala-D-Ala; 5 = L-Ala-D-Ala-L-Ala; 6 = L-Ala-L-Leu; 7 = D-Ala-L-Leu; 8 = L-Leu-L-Tyr; 9 = D-Leu-L-Tyr; m_1 = mixture of 1 and 2; m_2 = mixture of 1, 2, 4 and 5; m_3 = mixture of 3–5; m_4 = mixture of 6–9.

and Leu-Tyr diastereomers. On AWP the separation of L-Tyr-L-Arg from L-Tyr-D-Arg has been achieved notwithstanding the small difference (0.03) between their R_F values. In order to effect this separation, however, a 0.2-μl sample must be used.

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